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Modulating Fatty Acid Epoxidation vs Hydroxylation in a Fungal Peroxygenase

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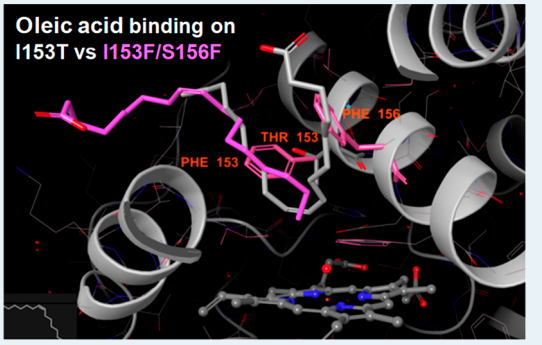
Supporting Information

ABSTRACT: Unspecific peroxygenases (UPOs) are fungal secreted counterparts of the cytochrome P450 monooxygenases present in most living cells. Both enzyme types share the ability to perform selective oxygenation reactions. Moreover, the *Marasmius rotula* UPO (*Mro*UPO) catalyzes reactions of interest compared with the previously described UPOs, including formation of reactive epoxy fatty acids. To investigate substrate epoxidation, the most frequent positions of oleic acid at the *Mro*UPO heme channel were predicted using binding and molecular dynamics simulations. Then, mutations in neighbor residues were designed aiming at modulating the enzyme epoxidation vs hydroxylation ratio. Both the native (wild-type recombinant) *Mro*UPO and the mutated variants were expressed in *Escherichia coli* as active enzymes, and their action on oleic and other fatty acids was investigated by gas chromatography−mass spectrometry in combination with kinetic analyses. Interestingly, a small modification of the channel shape in the I153T variant increased the ratio between epoxidized oleic acid and its additionally hydroxylated derivatives. A fully opposite effect was attained with the double I153F/S156F variant that completely abolished the ability of the *Mro*UPO to epoxidize oleic acid (while no activity was detected for the I153V variant). The rationale for these results was revealed by the substrate positioning in the above computational simulations, which predict a shorter distance between the oleic acid double bond and the oxygen atom of the peroxide-activated heme (compound I) in the I153T variant than in the native enzyme, promoting epoxidation. In contrast, the I153F/S156F double mutation fully prevents the approach of oleic acid in the bent conformation required for double-bond epoxidation, although its (sub)terminal hydroxylation was predicted and experimentally confirmed. The I153T mutation also increased the UPO selectivity on polyunsaturated fatty acid epoxidation, strongly reducing the ratio between simple epoxides and their hydroxylated derivatives, with respect to the native UPO.



KEYWORDS: *unspecific peroxygenase, Marasmius rotula, unsaturated fatty acids, epoxidation, hydroxylation, computational simulations, PELE software, gas chromatography−mass spectrometry*

INTRODUCTION

The so-called unspecific peroxygenases (UPOs, EC 1.11.2.1) are heme−thiolate enzymes secreted by fungi,[1] whose promiscuous oxygen transfer activities make them "dream catalysts" for difficult oxyfunctionalization reactions of industrial interest.[2] The cysteine ligand of UPO heme iron and the enzyme reaction chemistry are reminiscent of phylogenetically unrelated cytochrome P450 monooxygenases (P450s). Nevertheless, their extracellular nature and consequent higher stability, together with their self-sufficient monooxygenase activity (only requiring H_2O_2 to be activated), confer biotechnological advantages to UPOs.[1]

The first UPO was isolated by Ullrich et al.[3] from the basidiomycete *Agrocybe aegerita* (*Aae*UPO). Additional UPOs have been isolated from cultures of the basidiomycetes *Coprinellus radians* (*Cra*UPO),[4] *Marasmius rotula* (*Mro*UPO),[5] and *Marasmius wettsteinii* (*Mwe*UPO)[6] and the ascomycete *Chaetomium globosum* (*Cgl*UPO).[7] The classical chloroperoxidase from *Leptoxyphium fumago*[8] also belongs to the same protein family, although it exhibits low oxygenation and high halogenation activities compared with UPOs.[1]

Despite the limited number of isolated and characterized members, UPOs are widespread in fungi (and some funguslike organisms) with over 2000 *upo*-type genes identified in sequenced genomes and databases.[1,9] This constitutes a huge repertoire of potential biocatalysts with different oxygen

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transfer capabilities. However, until now only two *upo* genes from genomes/databases (corresponding to the basidiomycete *Coprinopsis cinerea* and the ascomycete *Humicola insolens*) have been heterologously expressed (by Novozymes A/S in *Aspergillus oryzae* host) and the recombinant enzymes (r*Cci*UPO and r*Hin*UPO) already evaluated for oxygenation reactions.[7,10−12]

Heterologous expression is required not only to explore the variety of UPOs in genomes, but also to understand the reaction mechanisms of these enzymes and to tailor their catalytic and operational properties for industrial biocatalysis. Apart from the work of Novozymes mentioned above, additional expression of wild-type *upo* genes as recombinant proteins has not been reported to date. A way to partially solve this limitation came from application of enzyme directed molecular evolution[13] to obtain mutated variants (such as r*Aae*UPO) tailored for expression in *Saccharomyces cerevisiae*,[14] which were later transferred to *Pichia pastoris*.[15] However, the evolved r*Aae*UPO obtained structurally differs from the wild-type enzyme, as shown by comparison of their crystal structures.[16]

As indicated by their systematic name, "substrate:hydrogen-peroxide oxidoreductases (RH-hydroxylating or -epoxidizing)",[17] hydroxylation and epoxidation are the main reactions catalyzed by UPOs. Widespread hydroxylation results in the introduction of alcohol, aldehyde/ketone, and carboxylic acid functionalities by subsequent hydroxylations and dehydration of the resulting *gem*-di/triols as shown for both aromatic[18] and aliphatic[19] compounds. The latter reactions include enzymatic activation of alkanes[20] and production of hydroxylated derivatives of industrial interest.[21]

On the other hand, both olefinic and aromatic compounds are epoxidized by UPOs to different extents. The latter epoxidized compounds easily rearomatize with introduction of a phenolic hydroxyl group.[22,23] However, different alkenes form stable epoxides with *Aae*UPO,[24] in an "activation" reaction of industrial interest.[25] Moreover, epoxidation of unsaturated fatty acids—a main constituent of soybean, linseed, and other plant oils—has high interest for the industrial production of a variety of chemicals and intermediates, including adhesive and binder components among others.[26] Industrial epoxidation of fatty acids is nowadays carried out by the Prileschajew[27] reaction via percarboxylic acids, generated using strong mineral acids as catalysts. To solve the drawbacks of this procedure, the use of the enzyme lipase generating peracids in the presence of H_2O_2 has been proposed.[28] However, this chemoenzymatic approach maintains the drawbacks related to peracid-based epoxidation.

Direct enzymatic epoxidation is catalyzed by P450s[29] and plant peroxygenases.[30] Nevertheless, problems associated with their low stability and frequent requirement for auxiliary substrates and/or enzymes prevent industrial implementation, in spite of numerous studies on their reaction mechanisms.[31−34] Therefore, the recently reported ability of some fungal UPOs to epoxidize unsaturated lipids could be the alternative of choice in the production of industrial epoxides. However, while many substrate oxygenation reactions are shared by different UPOs, fatty acid epoxidation is a characteristic of *Mro*UPO and *Cgl*UPO, while *Aae*UPO and *rCci*UPO do not epoxidize oleic and other unsaturated fatty acids.[35]

To understand and further improve the epoxidation activity of these enzymes, we heterologously expressed a wild-type (i.e., nonmutated) UPO for the first time, obtaining recombinant *Mro*UPO (r*Mro*UPO) as an active enzyme in *Escherichia coli*.[36] Then, guided by computational simulations of substrate diffusion and binding at the crystal-structure active site, we were able to engineer r*Mro*UPO to tune its epoxidizing vs hydroxylating activities on mono- and polyunsaturated (18-carbon) fatty acids.

MATERIALS AND METHODS

Computational Simulations. One of the solved crystal structures of *Mro*UPO (PDB 5FUJ, chain A) was used for molecular simulations. The system was optimized at pH 5.5 (*Mro*UPO optimal activity) with the protein preparation wizard from Schrödinger, resulting in histidines 81, 86, 102, and 131 being positively charged (doubly protonated), and the remaining being singly protonated at the δ position. The heme was prepared as the two-electron-oxidized compound I (C-I), a Fe^{4+}═O and porphyrin cation radical complex (see Supporting Information for the force field parameters for heme C-I). Heme charges in all modeling were obtained from a quantum mechanics/molecular mechanics (QM/MM) minimization using QSite,[37] at the DFT M06-L(lacvp*)/OPLS level of theory. In silico mutations were introduced using the side chain prediction routines in Prime.[38]

Three different simulation levels were used to describe the bound protein−ligand complexes. The simplest one involved substrate docking with Glide,[39] using default settings and the single precision SP option. Then, the new adaptive-PELE (Protein Energy Landscape Exploration) software[40] was applied to study substrate diffusion and local conformational sampling. The new protocol improves sampling by running multiple short simulations (epochs) where the initial conditions in each of them are selected through a reward function aiming at sampling nonvisited areas. PELE simulation involved 96 computing cores (trajectories) with 20 epochs of 12 PELE steps each. Interaction energies, in kilocalories per mole, were derived as $E_{ab} - (E_a + E_b)$, where E_{ab} is the total energy of the complex, E_a is the energy of the enzyme, and E_b is the energy of the substrate; all of them were obtained at the OPLS2005 level of theory with a surface GB implicit solvent model. Finally, molecular dynamics (MD) simulations were obtained with Desmond.[41] Initial structures (from Glide) were solvated and equilibrated with the default settings in Desmond. A 250 ns production trajectory was then produced with a Nosé−Hoover chain thermostat, relaxation time of 1.0 ps, and a Martyna−Tobias−Klein barostat with isotropic coupling and a relaxation time of 2.0 ps. The RESPA integrator was employed with bonded, near, and far time steps of 2.0, 2.0, and 6.0 fs, respectively. A 9 Å cutoff was used for nonbonded interactions together with the smooth particle mesh Ewald method.

Native Enzyme and Mutated Variants. Wild-type recombinant (hereinafter native) r*Mro*UPO and its I153S, I153T, I153V, and I153F/S156F mutated variants described below were expressed as soluble recombinant proteins in *E. coli* according to the method for native enzyme production, described in the patent under application number EP 18382514.[36] In short, the *mro*upo gene (with no introns) was cloned into the pET23a plasmid under the control of the T7*lac* promoter and transformed into *E. coli* BL21 cells. Bacteria were grown in ZYM-5052 media at 16 °C during 4 days. Then, cells were lysed by addition of lysozyme and sonication, and debris was removed from the soluble fraction

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by thorough ultracentrifugation. Three chromatographic steps—two anionic at pH 7.0 and one final cationic at pH 4.0—were used to obtain pure enzymes. The last purification step yielded electrophoretically homogeneous enzyme (as illustrated for r*Mro*UPO in Figure S1). Enzymes from the second chromatographic step were used when fully purified proteins were not required.

Proper folding and binding of the cofactor to the recombinant enzymes were assessed by inspection of the UV−visible spectrum of their resting state (Figure S2). Spectra were recorded using a Cary 60 spectrophotometer in 10 mM phosphate, pH 7.0. Formation of an adduct between the chemically reduced enzyme (ferrous form) and CO, typical of active heme−thiolate enzymes, was assessed in 250 mM phosphate, pH 8.0 (after addition of $Na_2S_2O_4$ and CO flushing) and used to estimate the enzyme concentration in fully and partially purified samples (Figure S3).[42]

The r*Mro*UPO variants were prepared using the Expand Long Template PCR kit from Roche (Basel, Switzerland) for site-directed mutagenesis. PCR reactions were run using the following DNA oligos harboring the desired mismatches (underlined nucleotides in bold triplets): (i) I153S mutation, 5′-CCGATTTAACTGCGACT**TC**CCGCTCTTCA-GAATCTG-3′; (ii) I153T mutation, 5′-CCGATT-TAACTGCGACT**AC**CCGCTCTTCAGAATCTG-3′; (iii) I153V mutation, 5′-CCGATTTAACTGC-GACT**GT**CCGCTCTTCAGAATCTG-3′; and (iv) I153F/S156F mutation, 5′-CCGATTTAACTGC-GACT**TT**CCGCTCTT**TC**GAATCTGCG-3′, along with their reverse complementary counterparts.

The PCR reactions (50 μL volume) were carried out in an Eppendorf (Hamburg, Germany) Mastercycler pro-S using 30 ng of template DNA, 500 μM each dNTP, 125 ng of direct and reverse primers, 5 units of Expand Long Template PCR System polymerase mix (Roche), and the manufacturer buffer. Reaction conditions included (i) initial denaturation step of 1 min at 95 °C; (ii) 22 cycles of 30 s at 95 °C, 30 s at 60 °C, and 7 min at 68 °C, each; and (iii) final elongation step of 7 min at 68 °C. The mutated *upo* genes were expressed in *E. coli* as described above.

Chemicals. The following unsaturated fatty acids (*cis* isomers) were obtained from Sigma-Aldrich: oleic (*cis*-9-octadecenoic) acid, linoleic (*cis,cis*-9,12-octadecadienoic) acid, and α-linolenic (*cis,cis,cis*-9,12,15-octadecatrienoic) acid. The following epoxide standards were used: (±)-*cis*-9,10-epoxyoctadecanoic acid and (±)-9(10)-EpOME (9,10-*cis*-epoxidized linoleic acid) from Santa Cruz Biotechnology, (±)-12(13)-EpOME (12,13-*cis*-epoxidized linoleic acid) from Cayman, and (±)-9,10−12,13-diepoxyoctadecanoic acid from Larodan.

As standards of epoxidized α-linolenic acid were not available, they were chemically synthesized. With this purpose, a solution of peracetic acid (1.8 mmol) and NaOAc (0.7 mmol) was added to α-linolenic acid (0.5 mmol) using a syringe pump for 1 h at 0 °C. The mixture was stirred for an additional hour at 0 °C and the products were recovered by liquid−liquid extraction with methyl *tert*-butyl ether resulting in a mixture of mono- and diepoxides.

Enzymatic Reactions. Reactions of the above fatty acids (0.1 mM) with r*Mro*UPO and its mutated variants (50−600 nM) were performed in 50 mM sodium phosphate (pH 5.5) at 30 °C and 30−60 min reaction time, in the presence of 1.25−5 mM H_2O_2. Prior to use, the substrates were dissolved in acetone and added to a final acetone concentration of 20% (v/v), although 40% was also tested with some compounds. In control experiments, substrates were treated under the same conditions but without enzyme. Products were recovered by liquid−liquid extraction with methyl *tert*-butyl ether, and dried under N_2. *N,O*-Bis(trimethylsilyl)trifluoroacetamide (Supelco) was used to prepare trimethylsilyl derivatives that were analyzed by gas chromatography−mass spectrometry (GC−MS) as described below.

Enzyme Kinetics. To study the kinetics of oleic acid oxidation, 1 mL reactions with 100 nM r*Mro*UPO and its mutated variants were carried out. Substrate concentration was varied between 12.5 and 1600 μM, and 20% (v/v) acetone was used as cosolvent. The reactions were initiated with 0.5 mM H_2O_2 and stopped after 3 min by vigorous shaking with 100 μL of 100 mM sodium azide. The time course of the reactions was previously analyzed to ensure that the kinetic parameters are estimated when the reaction rate is still in linear phase. All reactions were carried out in triplicate.

Product quantification was performed by GC−MS, as described below. Means and standard errors for the Michaelis constant (K_m) and turnover number (k_{cat}) values were obtained by nonlinear-least-squares fitting to the Michaelis−Menten model. Fitting of these constants to the normalized equation $v = (k_{cat}/K_m)[S]/(1 + [S]/K_m)$ yielded the catalytic efficiencies (k_{cat}/K_m) with their standard errors.

GC−MS Analyses. Chromatographic analyses were performed with a Shimadzu GC−MS QP2010 Ultra equipment, using a fused-silica DB-5HT capillary column (30 m × 0.25 mm internal diameter, 0.1 μm film thickness) from J&W Scientific. The oven was heated from 120 (1 min) to 300 °C (15 min) at 5 °C·min^{-1}. The injection was performed at 300 °C, and the transfer line was kept at 300 °C. Compounds were identified using authentic standards, by mass fragmentography, and comparing their mass spectra with those of the Wiley and NIST libraries. Quantifications were obtained from total-ion peak areas, using external standard curves and molar response factors of the same or similar compounds.

RESULTS AND DISCUSSION

***Mro*UPO Structure.** Although a publication on the crystal structure of *Mro*UPO has not been produced, two sets of atomic coordinates are available at the Protein Data Bank (PDB), as entries 5FUJ and 5FUK (Figure S4 illustrates the dimeric nature of the enzyme, and shows one of the heme access channels). Moreover, some structural features of this enzyme have already been discussed together with their use in oxyfunctionalization reactions.[6,43,44] Among them, the active-site access channel (Figure 1A), and the heme environment residues (Figure 1B) Glu157 and His86 helping the enzyme reaction with H_2O_2, Cys17 acting as the fifth ligand of the heme iron,[1] and the channel residues are relevant to explain the catalytic activities of *Mro*UPO on different substrates, including fatty acids.

Binding of Oleic Acid in *Mro*UPO. Binding of oleic acid in the computationally simulated C-I of *Mro*UPO, using the Glide and PELE softwares, showed analogous results: the unsaturated fatty acid accesses the heme channel in a fully bent conformation (Figures 2A and 3A). This contrasts with the binding of saturated stearic acid, analyzed for comparison, which adopted an extended conformation with ω-1 being the closest position to the oxygen atom of the C-I oxo group (Figure 2B).

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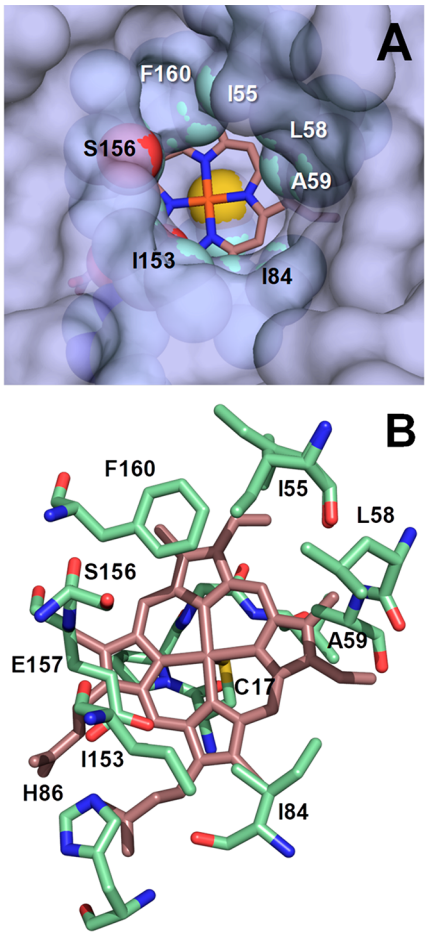

Figure 1. Axial view of the heme channel and environment in *Mro*UPO. (A) Semitransparent solvent access surface with residues forming the channel edge as van der Waals spheres and the heme cofactor as sticks. (B) Disposition of the heme cofactor and surrounding residues from the same orientation shown in (A). Residues are shown with CPK-colored atoms, while the heme cofactor is CPK-colored in (A) and pink in B (Cys17 sulfur is also visible as a yellow sphere in (A)). From PDB 5FUJ.

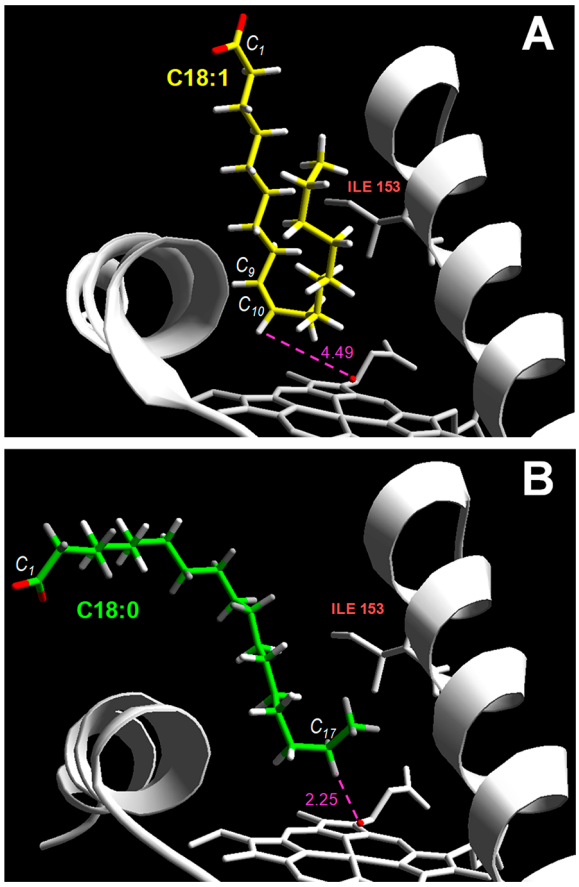

Figure 2. Initial Glide docking of oleic (*cis*-9-octadecenoic, A) and stearic (octadecanoic, B) acids (C18:1 and C18:0 as CPK-colored sticks with yellow and green carbons, respectively) on *Mro*UPO (detail of ribbon model) with indication of distances (in Å) between the C-I oxygen atom (red sphere) and the substrate C_{10} (A) and C_{17} (B) hydrogens. Ile153 to be mutated (see Figure 4) and heme cofactor are shown as white sticks.

A more detailed analysis of the oleic acid position inside the channel (i) identified Ile153 as a residue potentially limiting the approach of the substrate C_9═C_{10} double bond to the C-I oxygen atom to be transferred (Figure 2A), (ii) suggested that substitution of Ile153 by a slightly less voluminous residue (as illustrated in Figure 4A for the in silico-mutated I153T variant) could result in shorter Fe═O···HC_{10} distance (Figure S5) and improved epoxidation, and (iii) on the contrary, predicted that the double I153F/S156F mutation could abolish the epoxidation activity by preventing the approach of oleic acid in the required bent conformation (Figures 3 and 4). This is because, due to the introduction of two phenylalanines, the latter in silico variant has a narrower heme channel than the native *Mro*UPO and I153T variant (Figure S6).

Oleic Acid Reactions with r*Mro*UPO Variants. Simple (I153S, I153T, and I153V) and double (I153F/S156F) mutations—which could potentially improve or abolish, respectively, the epoxidation ability of *Mro*UPO (see above)—were experimentally introduced by site-directed mutagenesis. The mutated genes were transformed into *E. coli*, and the same protocol used for the native enzyme[36] was applied to produce the mutated variants. However, only I153T, I153V, and I153F/S156F could be obtained as soluble active enzymes and subsequently purified, with I153V lacking activity on oleic acid and other fatty acids. Interestingly, in *Aae*UPO and *Cci*UPO a threonine residue already occupies the position homologous to *Mro*UPO Ile153.[43] This agrees with the successful production of the I153T variant as an active enzyme, compared with the negative results obtained when a serine or a valine residue was introduced.

The I153T, I153F/S156F, and I153V variants were purified to homogeneity, maintaining the spectroscopic properties of native r*Mro*UPO (Figure S2). Then, their epoxidizing and hydroxylating abilities on oleic (Figure 5) and other unsaturated fatty acids were evaluated by GC−MS, as previously described for the wild *Mro*UPO isolated from fungal cultures.[35]

The most dramatic effect on enzyme activity corresponded to the I153F/S156F variant, whose reaction products (Figure 5C) were restricted to (ω-1) and ω hydroxy, keto, and carboxy derivatives of oleic acid (compared with the native enzyme epoxidized products shown in Figure 5A). The rationale for this result is illustrated by the PELE plots of substrate diffusion shown in Figure 6. The red spots show that the oleic acid C_{18} (ω position) easily attains catalytically relevant distances (at <4 Å from the C-I oxo group) with good interaction energies. Similar distances/energies are found for the more reactive C_{17}

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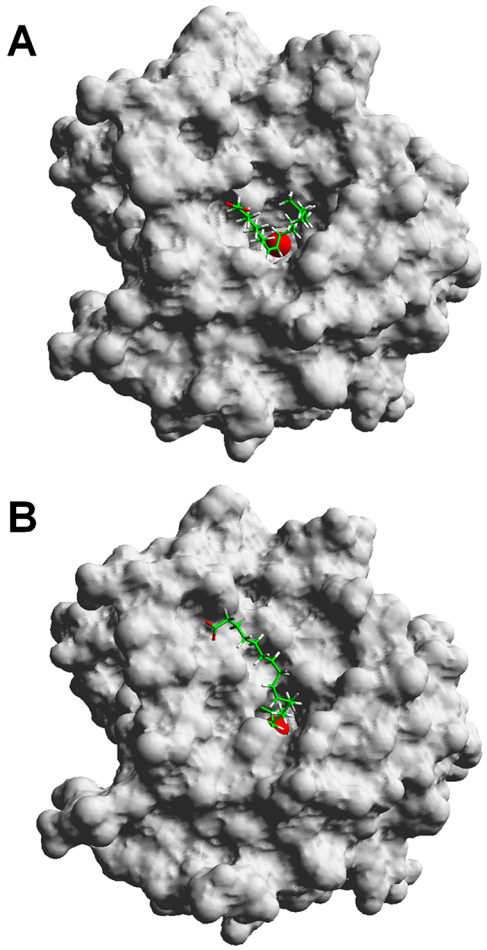

Figure 3. Solvent accessible surfaces of the I153T (A) and I153F/S156F (B) in silico variants of *Mro*UPO (monomer) showing, respectively, the bent and extended binding of oleic acid (CPK-colored sticks) at the heme access channel, as predicted by Glide, with the C-I oxygen atom at the bottom of the channel (as a red van der Waals sphere).

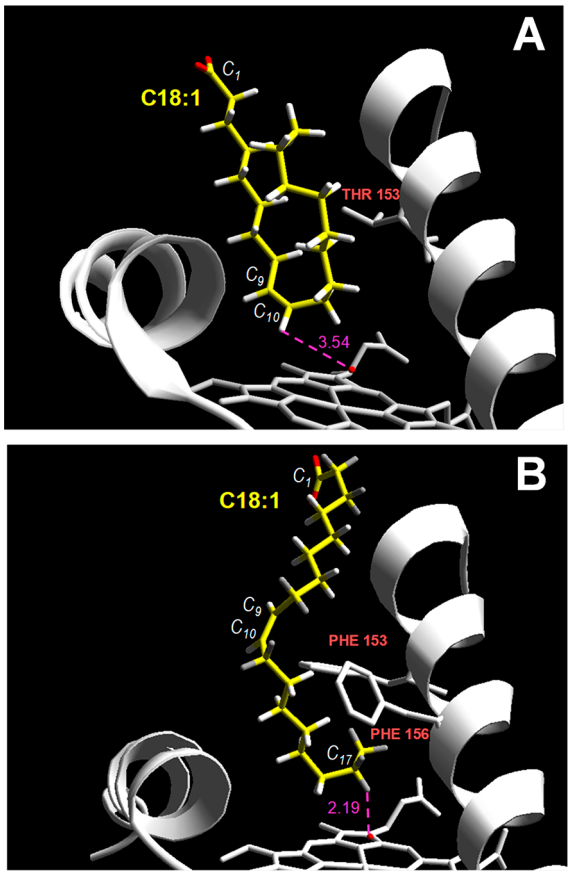

Figure 4. Initial Glide docking of oleic acid (18:1 as CPK-colored sticks) on the I153T (A) and I153F/S156F (B) in silico variants of *Mro*UPO (detail of ribbon model) with indication of distances (in Å) between the C-I oxygen atom (red sphere) and the substrate C_{10} (A) and C_{18} (B) hydrogens. Mutated residues (Thr153 in (A) and Phe153 and Phe156 in (B)) and heme cofactor are shown as white sticks.

(ω-1) position. This should favor (sub)terminal hydroxylation, in agreement with the experimental results. In contrast, the C_{10} (blue spots) atom always remains too far (>5 Å) and good interaction energies are only observed at long distances (>10 Å) from the C-I oxo group, preventing epoxidation of the double bond.

However, the most interesting effect observed is the improvement in the already described fatty acid epoxidation activity of *Mro*UPO,[35] as a result of the I153T mutation (Figure 5, parts A and B, respectively). Oleic acid epoxidation is observed in both cases, but its selectivity increased in the I153T reaction with significantly lower amounts of (ω-7)- and (ω-1)-hydroxylated epoxidized derivatives (Figure 5B) under the present conditions (the chemical structures of oleic acid products are shown in Figure S7, left). Due to the slight difference between the variants, for modeling justification we made use of (more precise) MD simulations this time.

The active site dynamics of oleic acid in *Mro*UPO and its I153T variant (Figure 7, parts A and B, respectively) show a significantly shorter average distance from the oleic acid double bond (between C_9 and C_{10}) to the C-I reactive oxygen in I153T, compared with the native enzyme. This agrees with the shortened distance (3.5 vs 4.5 Å) already observed in the initial docking (Figures 4A and 2A, respectively).

The modeling by PELE, addressing the ligand entrance into the active site (Figure S8), strongly supports the MD local sampling. The I153T variant presents a minimum at distances (oxo−C_{10}) of ∼3 Å, whereas *Mro*UPO presents the minima at distances of ∼6 Å, with a significant total energy increase at lower distances.

Reaction on Polyunsaturated Fatty Acids. Epoxidation of polyunsaturated fatty acids presents additional industrial interest since the introduction of several epoxy functionalities in the same molecule results in higher cross-linking potential. Therefore, linoleic and α-linolenic fatty acids were also evaluated as substrates of the enzyme (Figure 8).

Diepoxides (*syn* and *anti* isomers) and hydroxylated mono- and diepoxides are the main products of the r*Mro*UPO and its I153T variant reactions with linoleic acid (Figure 8, parts A and B, respectively). As in the oleic acid reactions, the amount of hydroxylated epoxides was lower with the I153T variant (the chemical structures of linoleic acid products are shown in Figure S7, center).

More interestingly, while a variety of other (additionally oxygenated) α-linolenic acid epoxidized derivatives were observed in the native r*Mro*UPO reactions (Figure 8C), nearly selective synthesis of diepoxides was obtained in the I153T

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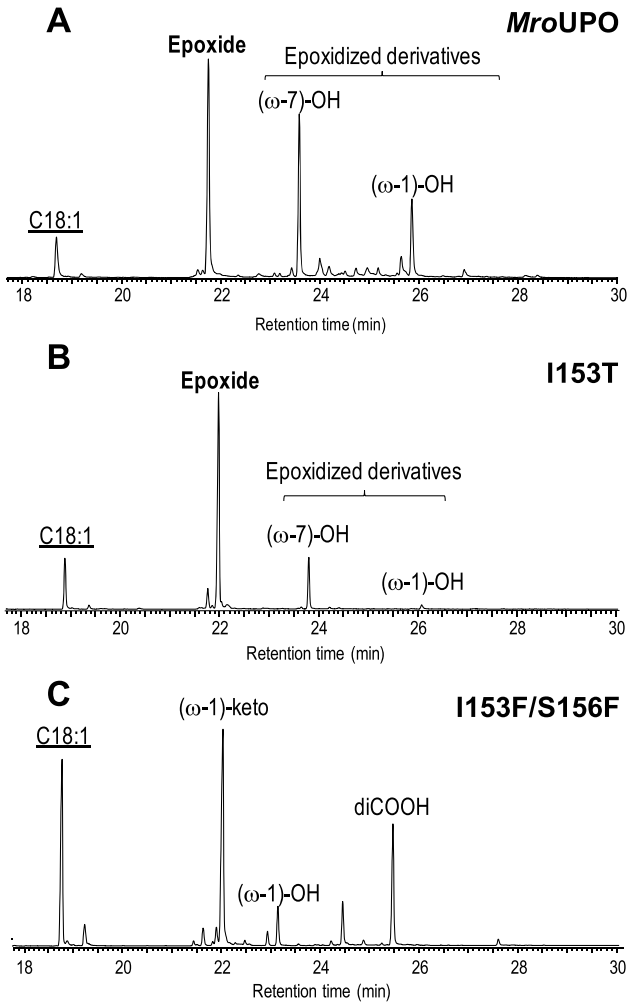

Figure 5. GC−MS analysis of oleic acid (C18:1, underlined) reactions with native r*Mro*UPO (A) and its I153T (B) and I153F/S156F (C) variants. Simple epoxides (bold) and additionally oxygenated epoxidized derivatives are the main products in (A) and (B), while only products from subterminal and terminal oxygenation are shown in (C). The reactions were performed with 100 μM substrate, 0.2 μM enzyme and 2.5 mM H_2O_2 for 30 min.

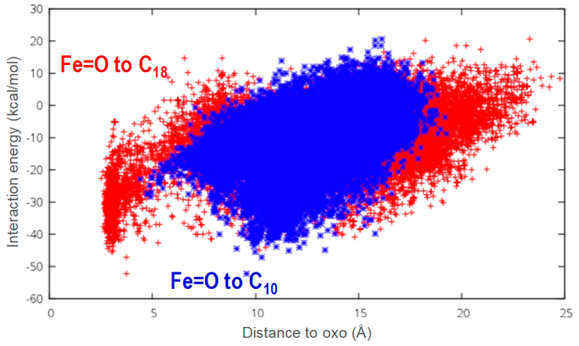

Figure 6. PELE diffusion of oleic acid on the I153F/S156F in silico variant of *Mro*UPO. The distances from substrate C_{10} (blue spots) and C_{18} (red spots) to the C-I oxo group are shown on the *x*-axis, and the corresponding interaction energies are shown on the *y*-axis.

reactions (Figure 8D) (the chemical structures of α-linolenic acid products are shown in Figure S7, right).

Kinetic Constants and Products. Despite difficulties in obtaining maximal (initial) reaction rates from chromatographic analyses of enzymatic reactions, the kinetics of oleic acid oxyfunctionalization by r*Mro*UPO and its two mutated variants could be analyzed in the range of 5−1600 μM substrate concentration (Figure 9).

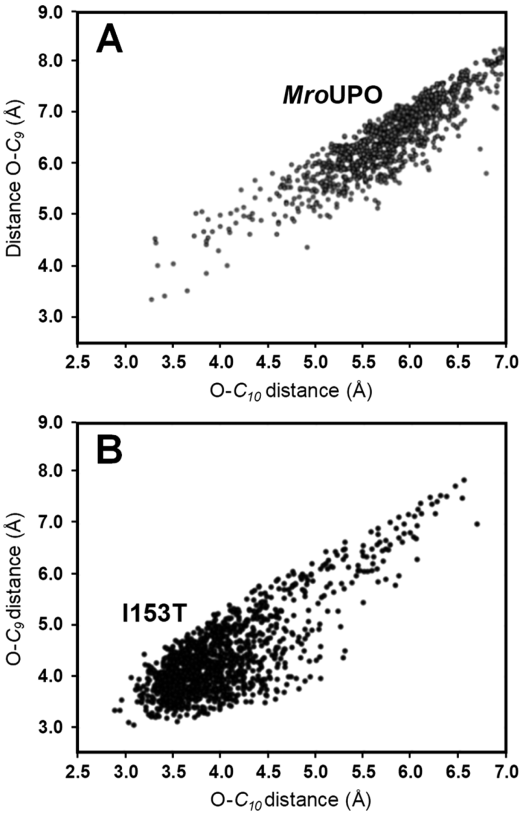

Figure 7. Positions of oleic acid after MD at the heme channel of *Mro*UPO (A) and its I153T variant (B) as shown by distances between the C-I oxo group and the C_{10} (*x*-axis) and C_9 (*y*-axis) atoms of the substrate.

The kinetic constants obtained (Table 1) revealed that the I153T mutation increases UPO catalytic efficiency (k_{cat}/K_m) epoxidizing oleic acid due to a significant improvement of the k_{cat} (from 1.5 ± 0.1 to 2.1 ± 0.1 s^{-1}). This is in agreement with computational simulations showing a shorter distance between the substrate double bond and the C-I oxo group (Figure 7), which will favor the electron and oxygen transfers. The experimental results also confirm the loss of epoxidizing ability and the new ω-1/ω hydroxylating ability of the I153F/S156F variant, predicted by PELE. Interestingly, the (sub)terminal hydroxylation activity by this variant (k_{cat} 2.3 ± 0.1 s^{-1}) is similar to the total oxygenation activity of the native r*Mro*UPO (k_{cat} 2.0 ± 0.2 s^{-1}) although lower than that of the I153T variant (k_{cat} 3.6 ± 0.1 s^{-1}) (Figure 9 and Table 1).

The time course of the epoxidation and additional oxygenation reactions (as shown in Figure S9 for the I153T variant) showed that the (ω-7)-hydroxylated and epoxidized derivatives of oleic acid are already present at the shorter reaction time (1 min), while doubly oxygenated (hydroxylated plus epoxidized) oleic acid appears later. Interestingly, reactions with a standard of epoxidized oleic (9,10-epoxystearic) acid failed to show hydroxylation at the allylic (C_{11}) position (data not shown), suggesting that (ω-7)-hydroxyoleic acid is the precursor of the doubly oxygenated derivative in the r*Mro*UPO reactions.

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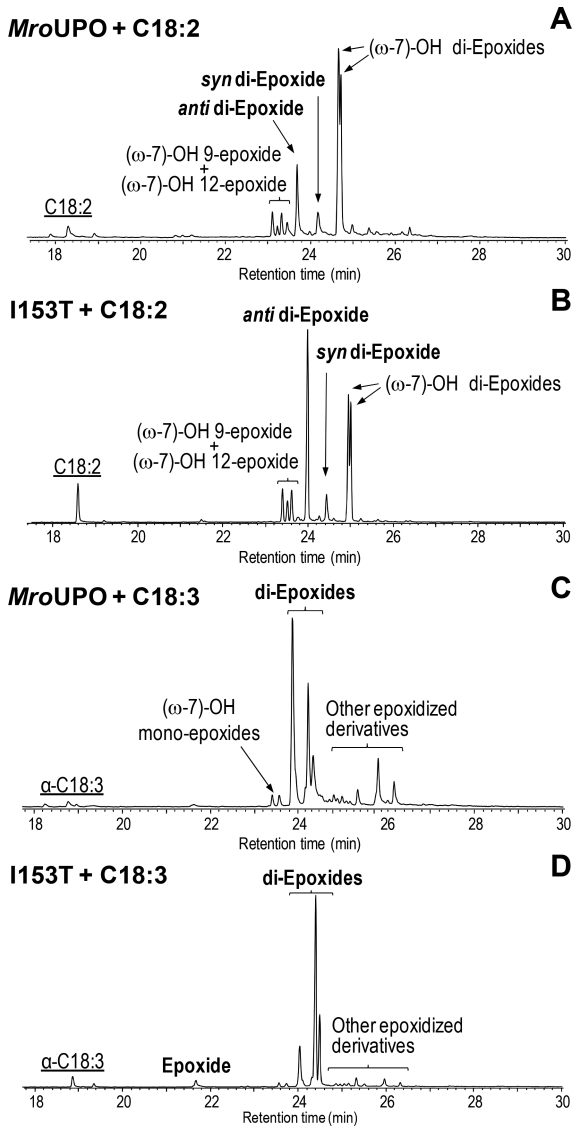

Figure 8. GC−MS analysis of reactions of linoleic (C18:2) and α-linolenic (C18:3) acids (substrates underlined) with native *Mro*UPO (A and C, respectively) and its I153T variant (B and D, respectively). Simple diepoxides (*anti* and *syn* isomers from linoleic acid in (A) and (B), and three unassigned isomers from α-linolenic in (C) and (D), bold) and other epoxidized derivatives are the main reaction products. The reactions were performed with 100 μM substrate, 0.6 (A and B) or 0.2 μM (C and D) enzyme, and 1.25 mM H_2O_2 for 30−60 min.

For quantitative comparison of the epoxidized products obtained, r*Mro*UPO and I153T reactions were performed under conditions (enzyme dose and/or reaction time) resulting in nearly complete (85−98%) conversion of the unsaturated fatty acids (Table 2). Then, the percentages of "only epoxidized" (mono- and diepoxides) and total epoxidized derivatives (including hydroxy- and oxoepoxides) were calculated. In these reactions, the I153T mutation increased *Mro*UPO selectivity epoxidizing the three fatty acids assayed, with the highest improvement (15-fold) with respect to native r*Mro*UPO being obtained for α-linolenic acid. In this way, up to 85% conversion of α-linolenic acid into diepoxy-octadecenoic acid was attained using the I153T variant of r*Mro*UPO.

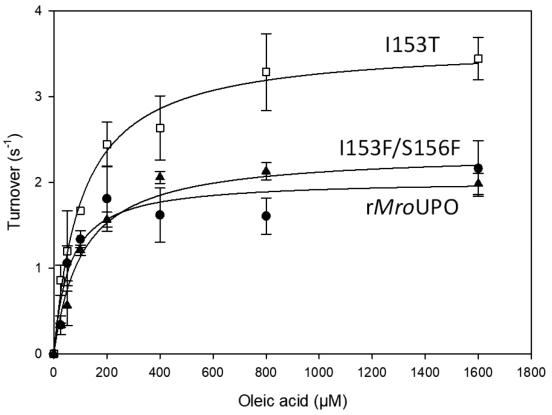

Figure 9. Comparison of kinetics of oleic acid reactions (total oxygenated products in Figure 5) with r*Mro*UPO (circles) and its I153T (squares) and I153F/S156F (triangles) variants.

Table 1. Kinetic Constants of r*Mro*UPO and Its I153T and I153F/S156F Variants in Oleic Acid Reactions Considering (i) All Epoxidized Products, (ii) Products Only Hydroxylated, and (ii) Total Oxygenated Products[a,b]

	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)
(i) all epoxidized products			
r*Mro*UPO	1.5 ± 0.1	52 ± 15	29 ± 8
I153T	2.1 ± 0.1	58 ± 8	36 ± 5
I153F/S156F	0	–	0
(ii) products only hydroxylated			
r*Mro*UPO	0.6 ± 0.0	61 ± 20	10 ± 3
I153T	1.4 ± 0.1	122 ± 17	11 ± 2
I153F/S156F	2.3 ± 0.1	108 ± 24	21 ± 5
(iii) total oxygenated products			
r*Mro*UPO	2.0 ± 0.2	64 ± 21	31 ± 11
I153T	3.6 ± 0.1	100 ± 13	36 ± 5
I153F/S156F	2.3 ± 0.1	108 ± 24	21 ± 5

[a]Note that only hydroxylation is produced by the I153F/S156F variant. [b]Means and 95% standard deviations.

Table 2. Molar Conversion, Percentages of Fatty Acid Derivatives (i) with Only Epoxides and (ii) Bearing Epoxides plus Other New Oxygenated Functions, and Epoxidation Selectivity, after Nearly Complete Conversion of Oleic (18:1), Linoleic (18:2), and α-Linolenic (18:3) Acids by r*Mro*UPO and Its I153T Variant (as Percentages of Total Chromatographied Products)[a]

		conversion	(i) only epoxides	(ii) other epoxides	selectivity[b]
18:1	r*Mro*UPO	94	40	57	0.67
	I153T	85	72	17	2.67
18:2	r*Mro*UPO	98	21	79	0.27
	I153T	93	42	58	0.72
18:3	r*Mro*UPO	98	44	56	0.79
	I153T	97	92 (4/88)	8	11.50

[a]Monoepoxide/diepoxide derivatives of α-linolenic acid are indicated in parentheses. [b]Epoxidation selectivity = ratio between only epoxides and other oxygenation products

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CONCLUSIONS

Based on computational simulations of oleic acid diffusion on the *Mro*UPO crystal structure, some mutations were designed to modulate the epoxidation vs hydroxylation activities of the enzyme, an important aspect to produce reactive compounds of industrial interest. Then, *E. coli* expression was used for the first time to produce UPO variants (bearing the previously designed mutations) which were obtained in soluble and active form. Finally, GC−MS analyses of unsaturated fatty acid reactions with the purified mutated variants confirmed their new/improved catalytic properties.

Using this combined approach, we show that the *Mro*UPO epoxidation activity on oleic acid can be removed by the double I153F/S156F mutation, which limits substrate entrance in the bent position required to approach its double bond to the reactive oxygen of the heme C-I. More interestingly, we also show that the epoxidation selectivity of the enzyme (on oleic, linoleic, and α-linolenic acids) can be improved by the I153T mutation that reduces the distance between the oleic double bond and the C-I oxygen atom to be transferred to the substrate.

In P450s, the ratio between epoxidation and hydroxylation activities has been associated with differences in the formation of enzyme reactive species (compounds 0 and I with hydroperoxo and oxenoid iron) involving an active-site threonine.[31,33] Strikingly, we report here that introduction of a threonine residue at the r*Mro*UPO active site results in more selective epoxidation. However, the effect of the latter mutation is due to more favorable positioning of the substrate (as shown by computational simulations) instead of an effect on the ratio between r*Mro*UPO reactive species (note that a glutamic acid, Glu157, plays in this UPO the role proposed for conserved threonine in P450s).[1]

In summary, we provide here the first example on UPO rational design (using *E. coli* expression) that hopefully will be followed by further protein engineering studies on other reactions of interest catalyzed by this and other fungal peroxygenases.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscatal.9b01454.

Purification of r*Mro*UPO; UV−visible spectra of r*Mro*UPO and its variants ; UV−visible spectrum of r*Mro*UPO complex with CO; general structure of *Mro*UPO; comparison of oleic acid in simulations; heme channel opening after in silico mutations; formulas of substrate epoxidized derivatives; PELE oleic acid migration in *Mro*UPO; initial time course of oleic acid reaction; methods including force field parameters of heme C-I and resulting spin density (PDF)

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Notes

The authors declare no competing financial interest.

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